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Use of Second Harmonic Generation (SHG) Imaging for 3-Dimensional Ultrastructural Visualization of Muscle Repair Mechanisms

Matthew Dufner
University of Connecticut - Storrs, dufner.matthew@gmail.com

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Honors Thesis

Matthew Dufner

Research Advisor: Dr. Morgan Carlson

Honors Advisor: Dr. James Cole

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Abstract

Sarcopenia, which involves age- and disease-related declines in muscle mass and muscle function, represents a very common and potentially reversible contributor to loss of function in older adults. Nevertheless, in spite of the immense clinical importance of this problem, the underlying pathophysiology remains poorly understand and few interventions other than exercise are available. Further, with regards to exercise guidelines, recent study (1) suggests that exercise itself may exacerbate muscle loss and degeneration, depending on age and variables such as sedentary lifestyle – thus physicians should not instruct their elderly patients to transition to exercise programs without careful patient history. Knowing the "state" of the muscle of a patient, such as via second harmonic generation (SHG), would be useful for developing patient-specific criteria.

Aging, disease and injury may all influence the number of nuclei in each muscle cell, as well as their size, shape and location within individual cells. These observable structural changes may represent underlying pathology or state of quality which would be able to serve as tools for early pre-clinical stage diagnosis. In this study, we have combined SHG imaging with various fluorescent dyes which are designed to stain nuclei and used a skeletal muscle injury and regeneration model to establish the ability of this approach to reliably and reproducibly evaluate the above nuclear parameters. By using the cobra cardiotoxin (CTX-1), which creates acute well defined injuries within the muscle, on the tibialis anterior (TA) and gastrocnemius hind limb muscles of mice, predictable and reproducible regenerative patterns (in response to acute injury) can be observed by harvesting muscle samples at specific time points during recovery. Through SHG imaging, we endeavor to document and analyze complete myogenic repair responses including (1) the mobilization of peripherally-located muscle stem cells (satellite cells) along myofiber peripheral lengths, (2) migration of satellite cell-derived nuclei toward central positions within the damaged myofibers and (3) peripheral migration of de novo nuclei within repaired myofibers for end stage repair. We expect this approach to further validate SHG as an accurate measuring technique for muscle repair and myonuclear movement. The longer range goal for these experiments is to translate our techniques toward clinical research and ultimately clinical care, in an effort to help older adults maintain function and independence.

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Acknowledgments

This work was carried out through the guidance of researchers at the University of Connecticut Health Center and the UConn Center on Aging; specifically, I would like to thank Dr. George Kuchel for accepting me into this research project and his invaluable guidance, Dr. Morgan Carlson for help developing the injury model and guidance with the physiological stages of muscle regeneration within the animal models along with his assistance in my thesis writing process. I would like to thank Dr. Bill Mohler for helping with the use of the SHG laser optical system and the analysis of the images obtained from the animal samples. Also, I thank Dr. Maren Fragala who guided the research and development using this SHG imaging and sample preparation approach during my previous research project at UConn Health Center and the Center on Aging. Additionally I would like to thank Dr. James Cole as my honors advisor for his assistance in the revision process.

List of Figures

Introduction

Muscle fibers have an unusual physiology: they are multinucleated cells that bond together to form myofibrils which together create the muscle fibers. Myofibrils themselves are created from the fusion of myoblasts, the original progenitor cells of the muscle. Myonuclei, the nuclei within the myofiber, are not able to divide and are dependent on satellite cells to replace their population. Satellite cells are the dominant stem cell population within muscles. Although other cell types have been described to contribute to muscle regeneration, their roles are relatively minor compared to that of satellite cells. Satellite cells lie outside the sarcolemma, the membrane of the myofiber, but within the basal lamina, the layer around the myofibers. These cells are normally nonproliferative and quiescent, however they activate in response to stimuli. To create additional myofibers and myonuclei, the satellite cells are activated and undergo self-renewal via asymmetric cell division to replenish stem cell pool. Some of the newly created satellite cells differentiate along myogenic lineage, giving rise to myoblast progenitors, myocytes and ultimately contribute to the formation of new myotubes. Within skeletal muscle, satellite cells form a small portion of the available nuclei; only around ~4% of the total nuclei present are satellite cells (2). Satellite cells are normally active in tissue homeostasis and maintenance, but are also activated by muscle injury to repair the muscle fibers or create new fibers.

Muscle repair follows a set route through regeneration with well characterized steps which can be divided into three distinct phases. Figure 1 shows a general depiction of myogenesis involved with the regeneration of skeletal muscle fibers. The first stage (Figure 1B) of muscle repair is the destruction and inflammatory phase, which occurs directly after the injury and starts with the activation of the satellite stem cells around the damaged area to create new myonuclei. At this same time, there is an invasion of macrophages and neutrophils that infiltrate the damaged area to begin the removal of the damaged fibers to prepare the area for the regeneration process. These cells enter the muscles through the circulatory system and remove the damaged muscle fibers and regions of the fibers. Without this process of removing the destroyed tissue, the regenerative process cannot continue at the same level. Once the damaged fibers are removed, the newly synthesized myoblasts are able to move towards the center of the new fiber (Figure 1C) and start the second phase where they begin to fuse together to form the multinucleated myotubes and scar tissue. After creation of the new tissue the reconstruction

phase can take place: the myonuclei that are centrally located migrate back towards the periphery (Figure 1D) and the myofibers are differentiated into the mature muscle fibers. The muscle fibers and scar tissue are remodeled and muscle function is restored (3). New fibers can be synthesized within the basal lamina of the previous damaged (what?) using this hollow shell as a scaffold to form the new fibers within. Alternatively, if the region was so damaged that even the basal lamina of the cell had to be removed, the myoblasts can fuse without the scaffold and form a completely new fiber.

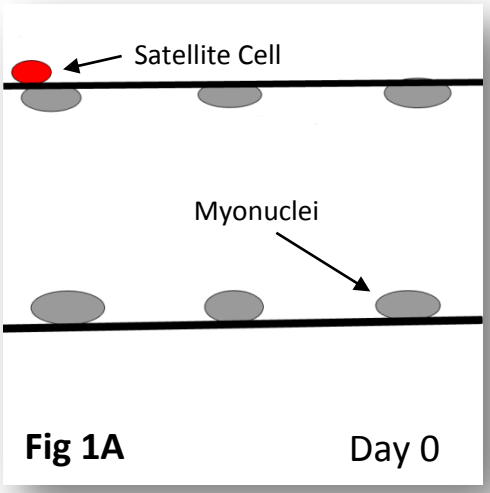


Fig 1A Day 0

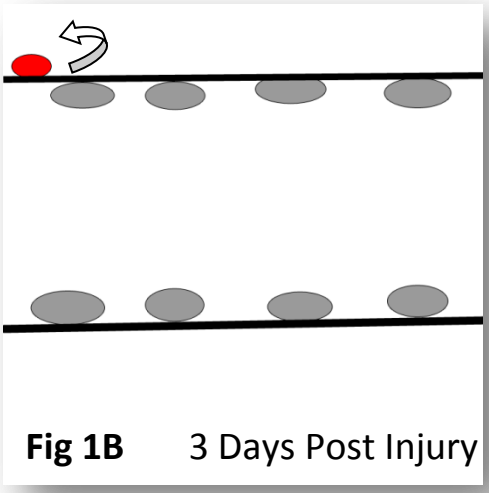


Fig 1B 3 Days Post Injury

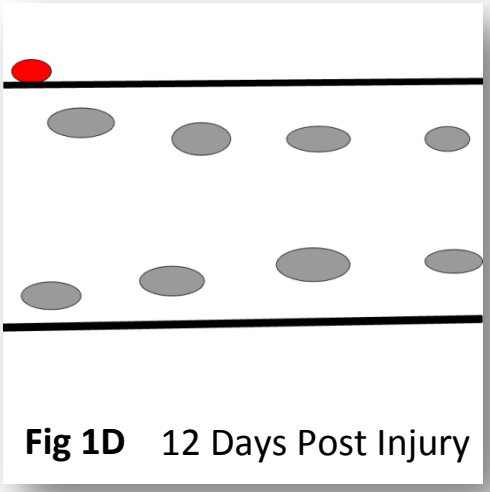


Fig 1D 12 Days Post Injury

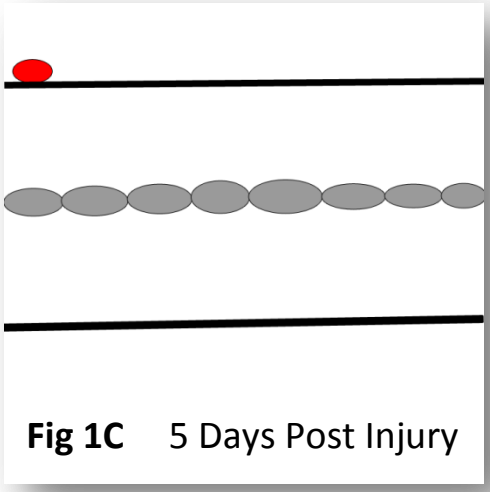


Fig 1C 5 Days Post Injury

Figure 1- General Muscle Repair and Myogenesis- (A) Before injury to the skeletal muscle, the satellite cell (red) is quiescent and remains on the edge of the muscle fiber, between the basal lamina and the sarcolemma. (B) After injury, the satellite cell begins to rapidly proliferate undergoing self-renewal and creating additional myonuclei. This establishes an increased myonuclei population while maintaining the satellite cell population. (C) By 5 days post injury, the myonuclei created by the satellite cell have migrated towards the center of the damaged fiber to begin creating the new myofiber. (D) 12 days after the injury, the muscle is returning to its normal resting state. As the regenerative process finishes, the myonuclei that were in the center of the fiber migrate back out towards the periphery of the fiber where they will remain.

Declines in muscle mass and function represents a significant contributor towards the loss of function in older adults (4). Sarcopenia is the degenerative loss of muscle that is associated with aging and disease. Even though this condition has a major clinical significance, the pathophysiology of sarcopenia is not well understood with few treatment methods other than exercise available (5). One of the major obstructions is the lack of a deep understanding of the mechanisms behind sarcopenia resulting from the lack of suitable techniques to provide quantitative assessments of the ultrastructure changes involved in sarcopenia such as changes in the sarcomeres, nuclei or mitochondria. Second harmonic generation imaging may provide a technique that provides numerous advantages over electron microscopy techniques for the study of muscle ultrastructure.

Second harmonic generation imaging is a non-linear optical tool that is based off of a principle of frequency doubling and requires a highly polarizable material with non-centrosymmetric molecular organization. With SHG an intense laser field creates a nonlinear polarization with the molecule to produce a coherent wave of exactly twice the incident frequency. SHG is dependent on the non-centrosymmetric nature of the molecule being visualized; the SHG waves that are created through the frequency doubling are vector quantities, and centrosymmetric molecules would result in waves of equal and opposite polarization, canceling each other out, and so producing no signal (6). This principle allows us to use the lower energy infrared light and merge them into a visible photon at twice the energy; since there is no excited state, no energy is lost (7). The myosin of the thick filaments within the sarcomeres is a perfect example of a molecule that can be illuminated through the use of second harmonic generation (8). Collagen also is able to emit a strong signal with SHG. Both the large

components of muscle tissue, the sarcomeres through the myosin filaments and collagen, are able to be imaged through SHG microscopy. This makes SHG able to provide a detailed mechanism to observe the structure and integrity of muscle fibers. It was seen that striated muscles provide a significant source of SHG, and SHG proved to be an optimal imaging technique to image deep sections of the tissue. SHG is a nonlinear form of excitation and so, signal comes only from the point of focus. Near infrared has less scattering than visible light, and provides an improved resolution within deep tissue. SHG provides a variety of benefits besides its high resolution within deep tissues and its lack of required dyes or staining. One advantage is that extensive preparation dealing with fixation, staining and sectioning is not required for SHG. Additionally, no cytotoxic or phototoxic effects from exogenous labels have been observed. Since SHG reacts with the endogenous structures within the tissue it is applicable to all species and is not limited by genetic mutants such as GFP or staining through dyes, which makes it an ideal candidate for medical imaging (9). Electron microscopy (EM), a well-established approach for qualitative ultrastructural analysis, is subject to development of contraction artifacts and is ill-suited to quantitative analysis because each ultrathin section samples only a minor portion of each cell. SHG instead is able to sample the entire muscle section. Errors can easily be propagated and if the sampling is biased at any stage of the process, the values will deviate away from their true value even if large amounts of data are acquired (10). By sampling an entire block of tissue it removes a possible source of error that can help to reduce deviation from the true value, making it a valuable quantitative tool. While SHG does not require a stain of its own, it is easily combined with fluorescent labels to provide additional parameters to be monitored and analyzed

Materials and Methods

In this study experiment, the cobra cardiotoxin (CTX-1) was injected into sites within the tibialis anterior (TA) and the gastrocnemius to provide an acute injury (11). CTX-1 induces tonic contracture of the muscle fibers by affecting a membrane calcium binding site, causing irreversible damage to the fiber.(12) This injury provides a predictable and reproducible regenerative pattern that could be imaged using SHG along with fluorescent probes to stain the nuclei within the muscle fibers. The mice were sacrificed at three time points besides the 0 day control to capture different stages of the regenerative process. From each time point, the TA and gastrocnemius was removed and fixed in a solution of 4% paraformaldehyde. Prior to imaging,

each sample was stained for nuclei with a 5µg/ml solution of Hoechst for 5 minutes before being placed in a solution of 50%glycerol/50% PBS. While being imaged, the sample was placed on a glass slide while suspended in PBS to keep it from dehydrating during the imaging process. Two Ti:Sapphire Spectra Physics Mai Tai DeepSea femtosecond pulsed lasers were used to emit a 100 femtosecond pulse of 900 nm light at the sample so that the 450 nm emitted light can be isolated. Four Chroma bandpass filters were used, 435-485 nm, 500-550 nm, 570-620 nm and 640-680 nm; the relative range for each filter allows the collection of nuclei in one channel and the 450 nm SHG signal in a separate channel with minor levels of overlap. Each image stack obtained from the muscle sample was isolated by scanning through the sample for key physiological markers such as central nuclei, high densities of nuclei or damaged muscle fibers which are indicative of the acute damage zone created through the injection of CDX-1. Images were captured using a 20x zoom lens with a 1.25 magnification of the image recorded. The Hoechst stain relies on two photon fluorescence to illuminate all nuclei within the sample and visualize major components of the muscle tissue's regenerative process. Staining for nuclei was carried out to observe additional characteristics of the muscle's regenerative process in this experiment, and was not required to capture the images using the second harmonic generation imaging system.

From each time point taken in this study, a different key process in the regeneration of the muscle fibers can be seen. In the 3 day post injury time point the satellite cells are proliferating and the macrophages and neutrophils are rapidly infiltrating the tissue, creating a chaotic appearance in the samples (Fig. 2b). Since the Hoechst stain used for these images reacts by binding to DNA, it reacts with all nuclei present within the sample, illuminating the satellite cells, myonuclei, and the many infiltrating cells that are part of the normal acute inflammatory event typical in such damage; no differentiation between the cell types can be distinguished from this stain alone. In the 5 day post injury samples, the satellite cells have created a large number of myonuclei which have moved to the center of each damaged fiber to repair the injured areas or to synthesize new strands. In Fig. 2c a field of denovo fibers is being synthesized without using a scaffold left over from the previous damaged fibers. At the stage of 12 day post injury (Fig. 2d), most of the nuclei are at the periphery again or migrating from their central location. At this time point the satellite cell-derived cells are still enlarged from their previous state, but are returning to their normal basal state as the repair process ends.

Results

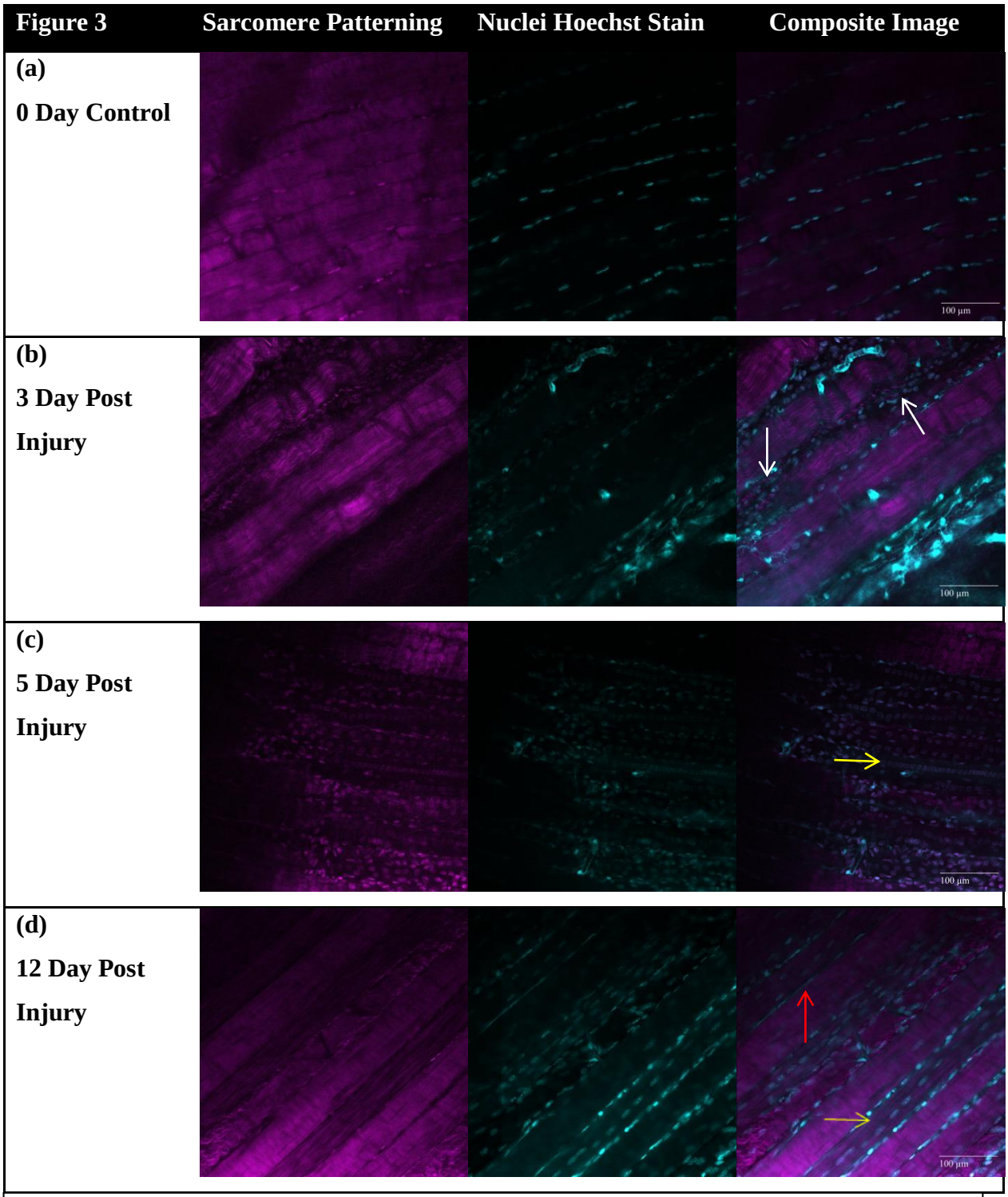


Figure 2-Time Points of Muscle Regenerative Process- This figure shows slices of samples taken from each time point at 20x magnification. (a) 0 day sample, showing the normal state of the muscle; nuclei remain on the periphery of the fibers in a well ordered distribution in moderate numbers. (b) 3dpi muscle showing proliferation from the satellite cells and influx of macrophages and neutrophils. The white arrows indicate regions of high concentrations of nuclei. (c) 5dpi showing an area of denovo fiber synthesis (yellow arrow) and the new myonuclei in central locations. (d) 12dpi showing some nuclei still in central positions (yellow arrow) but the fiber is returning to the normal resting state (red arrow). Muscle tissue is expected to have returned to its full resting state by 20-30 days post injury.

In the following sections snapshots are taken to illustrate mechanisms of muscle repair that have resulted from the acute injury of the CTX-1. The snapshots of the regenerative mechanisms are taken from multiple time points, and multiple muscle samples. The properties that are highlighted in this section are not unique to the cardiotoxin used in this study, and are found in all muscle regeneration.

Nuclei Repair through (i) Fusion and (ii) Denovo fiber synthesis

From the muscle samples collected both methods of regeneration that the myonuclei can use have been seen. In Figure 3a denovo synthesis can be seen, where the muscles were so damaged that the myonuclei had to recreate the fibers without the preexisting scaffolding of basal lamina. However, a more common regenerative method is to simply repair small regions of damage. This can be seen in Figure 3b and is a more typical response to small injuries and physiological myotrauma as a consequence of tissue homeostasis/maintenance requirements. The more routine repair process can be seen at a small scale in Figure 3b. This occurs as the myoblasts in the center of the fiber fuse together forming myotubes in continuity with the preexisting fibers of the muscle as continuous regeneration. Alternately, if there is complete degradation of the fiber but the basal lamina still remains intact, the myoblasts can synthesize new myofibers within the preexisting basal lamina, a process which is known as discontinuous regeneration (13)**.**

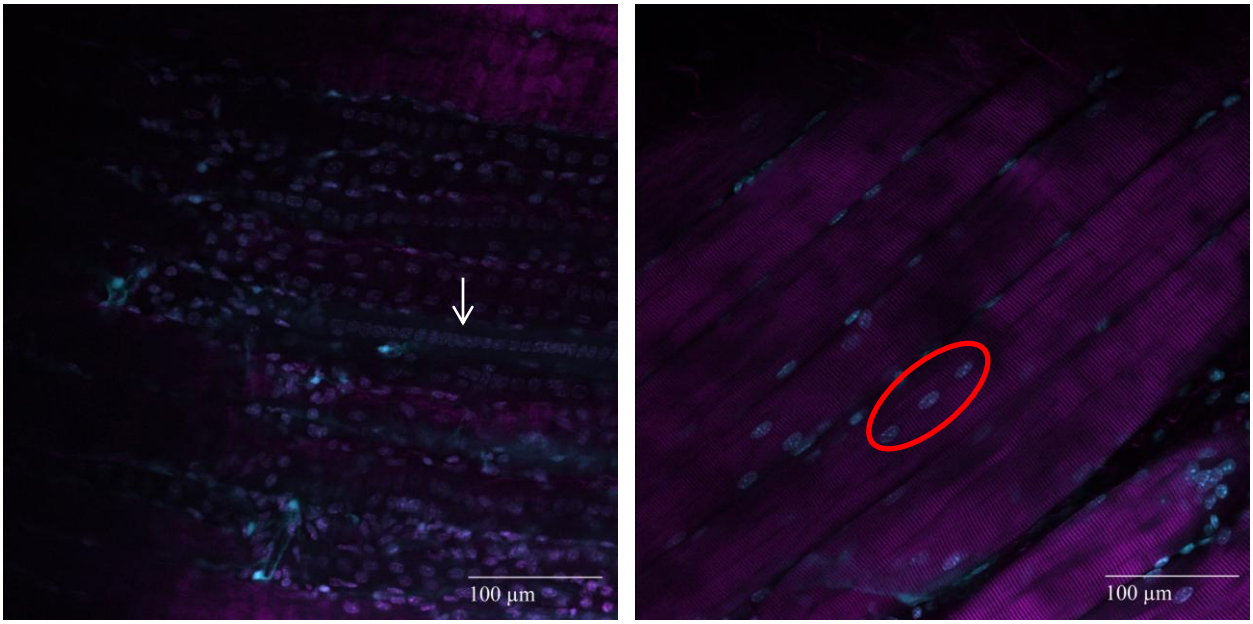


Figure 3-Nuclei Repair Through Fusion and Denovo Fiber Synthesis-(a) Shows denovo synthesis of fibers not relying on the emptied basal membranes of the previously damaged muscle fibers. (b) A small example of repair to a fiber through the fusion is circled in red. This happens when a few myonuclei migrate to a damaged region to fuse to the preexisting fiber to replace the damaged area instead of large scale building of the fibers seen in (a). Both images are at 20x zoom.

Satellite Cells

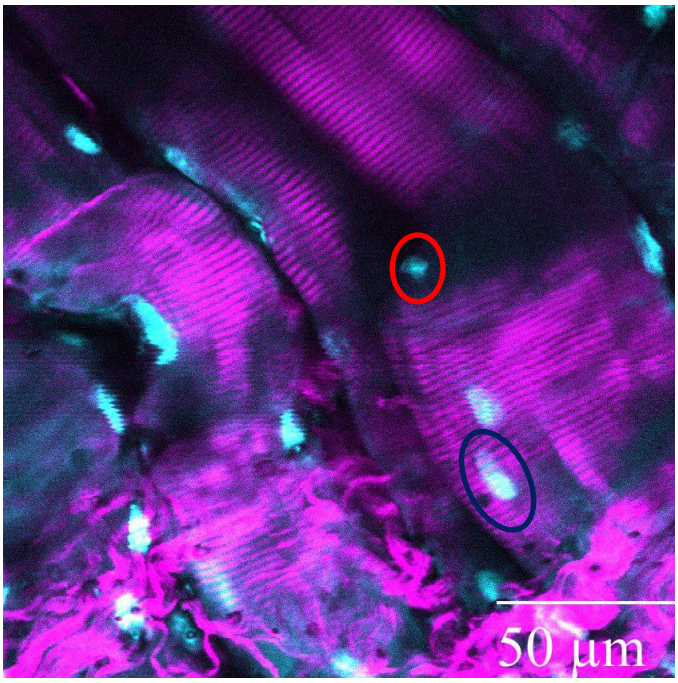


Figure 4-Putative Satellite Cell

In this figure, a comparison between the size of satellite cells which average 10µm and the myonuclei which are 30µm. Satellite cells can be found on the outside of myofibers and account for only ~4% of the nuclei present in mature myofibrils. A putative satellite cell is circled in red on the image while a typical myonuclei is circled in blue.

Satellite cells are much smaller than the more common myonuclei. In Figure 4 the circled nuclei is most likely a satellite cell due to its small compact size and relative position compared to the larger myonuclei present within the fibers. In skeletal muscle, satellite cells usually are approximately 10 μm while the myonuclei are around 30 μm. The size difference between satellite cells and myonuclei a major defining characteristic of the satellite cells, and is a useful tool for finding satellite cells besides depending on their location between the basal lamina and the sarcolemma. Satellite cells are not the common cell within the muscle fibers; instead they occupy a relatively small portion of the resident cells as in mature adult tissues they occupy only ~2-4% of the nuclei present within the muscle fibers (14). While putative satellite cells can be identified through the use of the Hoechst stain, additional marker stains are required to confirm sublaminal position and contact with the sarcolemma such as laminin and dystrophin.

Nuclear Shape

Satellite cells are normally quiescent within the muscle cells. After injury to the muscle, the satellite cells activate so they can produce new myoblasts to replenish the muscle fibers and regenerate the surrounding muscle structure. This activation of the muscle cells can be seen as an increase in size and number of the nuclei as they differentiate along myogenic lineage into specific cell types, i.e. myoblasts and myocytes. Satellite cells are normally characterized by a high nuclear to cytoplasmic ratio along with dense heterochromatin, but as they activate the cells expand to begin proliferating. When comparing the size of nuclei from the 0 day (Figure 5a) resting mouse and the 12 day post injury sample (Figure 5b) it can be seen that the nuclei are returning from an enlarged state back to their original size. As the muscle fiber matures the myonuclei on the periphery are condensed and compressed providing an almost squashed appearance where as they were actively regenerating the fiber they took on a more rounded shape outside of their normal location.

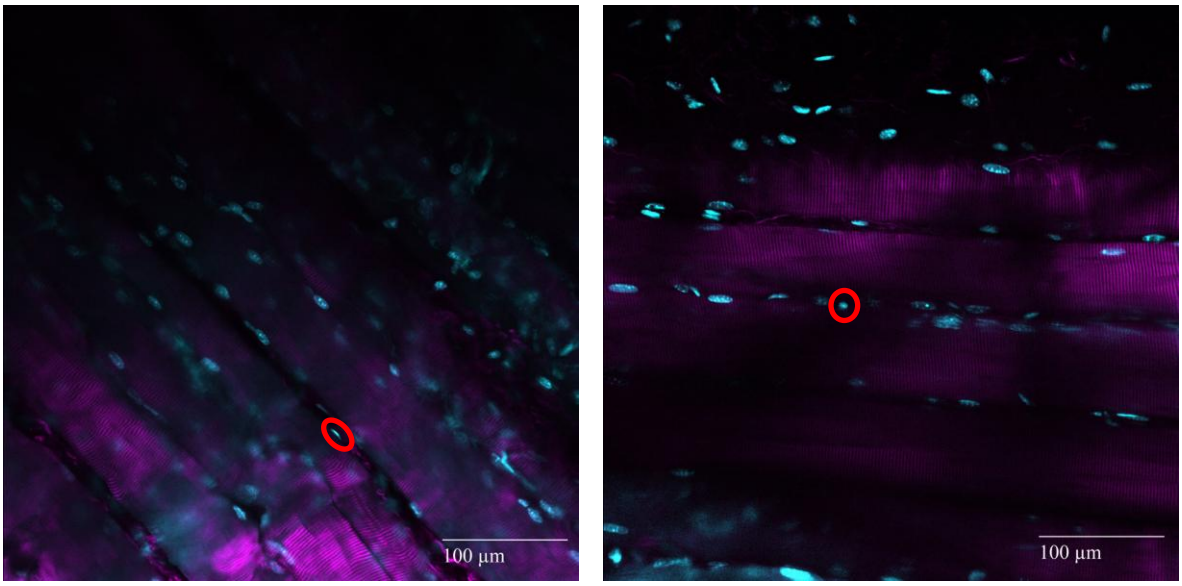


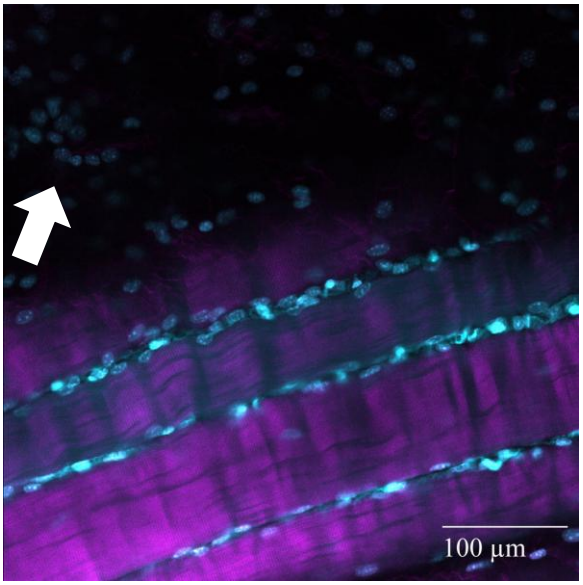
Figure 5-Nuclear Shape of 0 day and 12 dpi- This figure shows potential satellite cells circled in red. The first image shows a potential satellite cell from the 0day control sample while the second image is from 12 days post injury. The satellite cell for the 12dpi has a larger appearance resulting from the recent regeneration within the fiber. As time progresses it returns to the smaller, almost squashed appearance the 0day image presents.

Images were captured at 20x magnification.

Niche Structure.

Muscle fibers are not alone within the muscle tissue, microvasculature and extracellular matrix can be seen in regions, especially where damage and inflammation has occurred. The extracellular material present may remain in the tissue well past when the inflammatory response ends; cavities of the material can form pockets between the muscle fibers. Veins are an important component of the regenerative process and normal muscle function and infiltrate the region along with nerves in response to injury (3). Especially in the regeneration process, blood vessels are critical to transport the required cells to the damaged regions of the fiber. Within the extracellular material there is a host of other cells which can all be seen by the nuclei stain used in these images. Figure 6 shows a region of extracellular material swarming with nuclei. The myonuclei and satellite cells normally present within the muscle fibers do not migrate to locations within the extracellular material and are limited to within the fibers themselves; because of this limited range, all the nuclei seen within the extracellular material are largely from

exogenous cells such as macrophages, neutrophils and other cells invading the damaged region of the muscle.



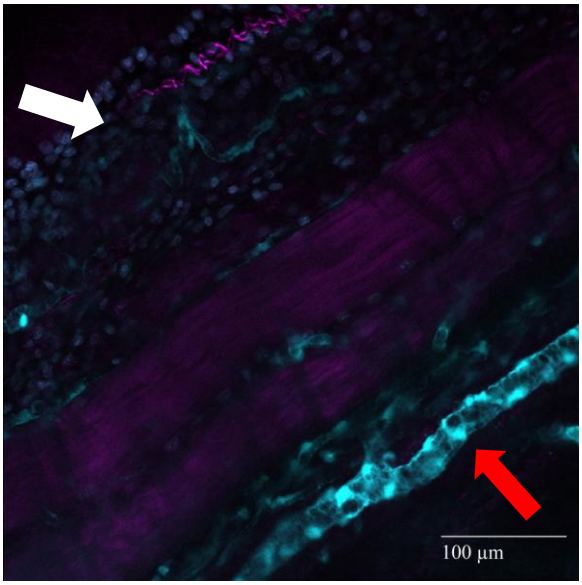


Figure 6-Niche Structure of Muscle Fibers- In both (a) and (b) extracellular materials can be seen with a host of nuclei within it (white arrows). These nuclei cannot be myonuclei or satellite cells from the muscle fibers and are instead invading cells. In (b) a blood vessel can also clearly be seen to the right of the fiber identified by the red arrow; blood vessels play an important role in muscle fibers and especially in the regenerative process in damaged muscle tissue.

Both images were captured at 20x zoom

Interstitial Expansion

As the macrophages and neutrophils infiltrate the damaged tissue they cause a series of reactions within the muscle tissue that help advance the degeneration of the tissue and the recruitment of myoblasts. Macrophages initially are restricted to phagocytosizing the necrosed tissues within the fiber, but later enhance the growth of myoblasts within the region by releasing myogenic growth factors (15). Macrophages and neutrophils release cytokines as they enter the damaged region of tissue that help to recruit additional macrophages and neutrophils but also help to attract nearby satellite cells to the damaged region. This response allows the targeting of the cells towards the damaged region; additionally the permeability of blood vessels is increased, allowing for greater transport of proteins and fluid into the damaged region(16). This

accumulation within the damaged region along with the production of extracellular material by the cells can create regions between the muscle fibers that can remain enlarged through the repair process. These regions along with adipocytes and scar tissue are vital to the regenerative process. In Figure 7 this interstitial expanse can be seen, and compared to the nearby fibers which have remained or returned to the more normal dense packing present in muscle fibers. As muscles degenerate, the normal balance between regenerated fibers and adipocytes and scar tissue (fibrosis) shifts more towards the formation of adipocytes and scar tissue. This shift can lead to major physiological changes and can be seen in some extreme examples in muscular diseases. Duchenne muscular dystrophy is one such disease where the adipocytes and scar tissue are the dominant tissue in the region instead of healthy fibers. This increased amount of fibrosis and adipocytes is associated with a loss of muscle strength and function (17).

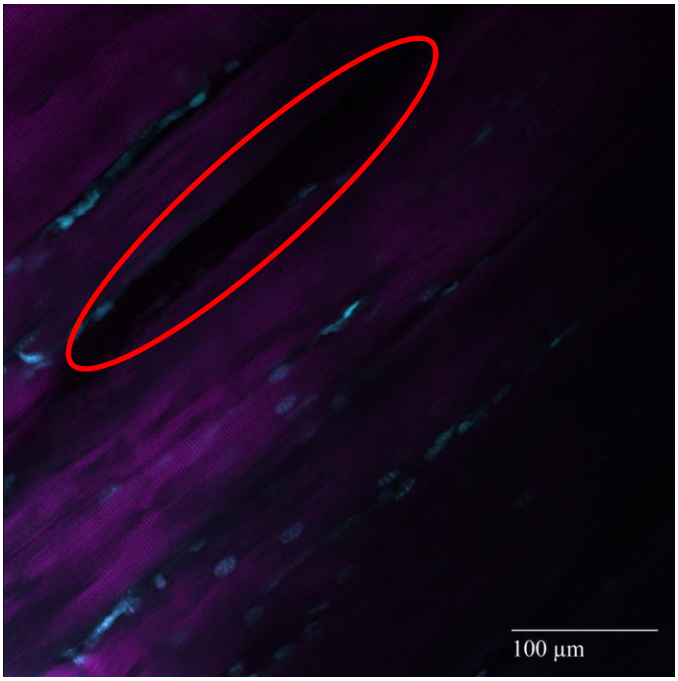


Figure 7- Interstitial Expansion Between Muscle Fibers

The interstitial region between fibers is circled in red in the figure, showing the large area that was created between the two neighboring fibers. Especially in the lower region of this expanse wisps of material can be seen that will remain in this region, even if the fluid drains. These regions of occupied space may result in a decrease in muscle strength as they occupy regions of the muscle tissue that could otherwise have muscle fibers occupying them.

Heterogeneity of Regeneration

The regenerative process and the movement of nuclei within fibers is not fully synchronized between each fiber or damaged region, it is instead independent for each local region or fiber. The fact that this process is not all fully synchronized can clearly be seen in Figure 8 where multiple stages of nuclei placement from the regenerative process can be seen at once. Nuclei can be seen still in their centrally located state where they are creating new fibers or

repairing damage, but nuclei can also be seen migrating away from their previous center location back towards the periphery of the muscle fiber. Their final destination, the periphery of the cell, also hosts some nuclei, showing almost all stages of nuclei migration within a small area of muscle fiber. (Figure 8)

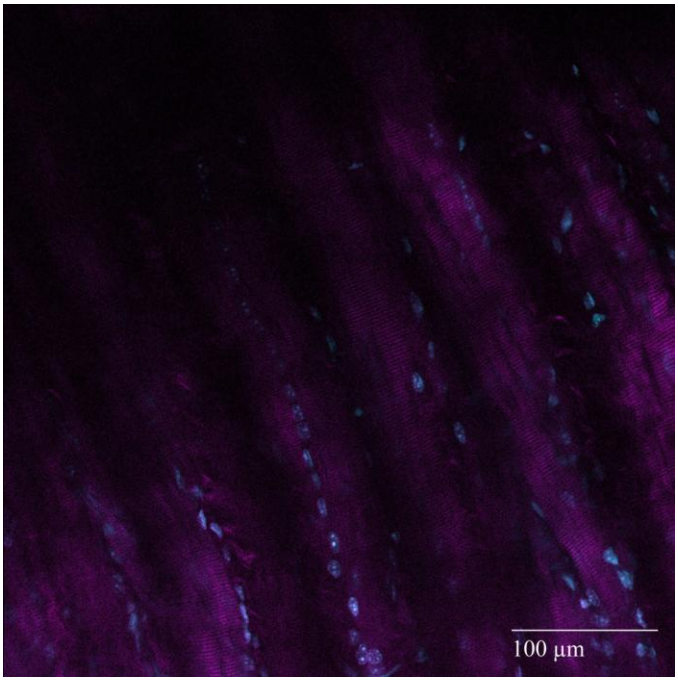


Figure 8-Heterogeneity of Regeneration

Within this one frame of a section of muscle nuclei can be seen in different stages of migration within the fibers. In the bottom left nuclei can be seen at the periphery of the fibers which in the central fiber the nuclei are still centrally located. Towards the right side of the figure nuclei can be seen finishing their migration towards the periphery of the fiber against the sarcolemma.

Imaged at 20x magnification.

Damage Interface

Damaged areas within muscle tissue do not necessarily act on a gradient within the fibers, in some cases clear divides can be seen of the interface between damaged tissue and healthy tissue. In Figure 9 this can clearly be seen between a region of denovo fiber synthesis and a relatively healthy region of fibers. In the top section there remains a fiber showing fully striated regions with no central nuclei evident of recent repair and no macrophages or neutrophils present to degrade tissue. Immediately adjacent to that fiber however it can be seen where degradation has claimed entire fibers, requiring myoblasts to create new fibers instead of relying on the empty basal laminas as scaffolds. This is an interesting phenomena, showing that the damage is evaluated by each fiber and not as the muscle tissue as a whole. This is also a perfect example of how targeted the regenerative process is within the skeletal muscle: only the fibers that have sustained large amounts of damage are replaced and the rest have only the small areas repaired.

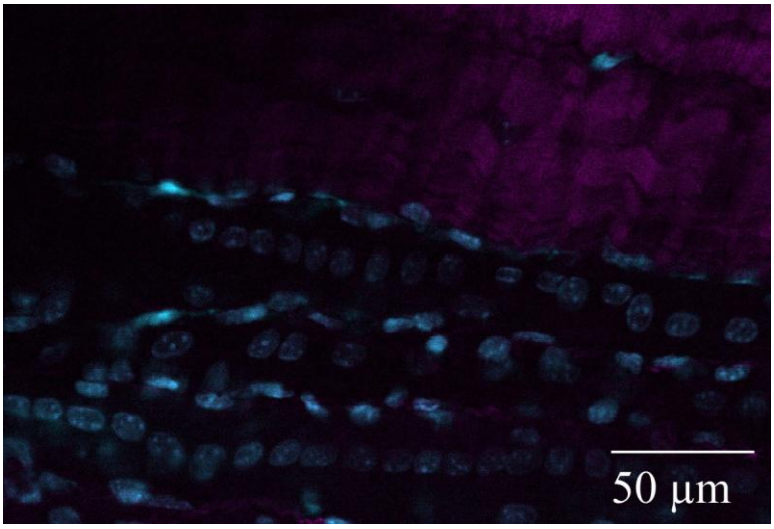


Figure 9-Damage Interface

This figure shows the interface of a healthy fiber with an area of completely denovo fiber synthesis that can be identified by the nuclei lining up in their central locations to create a new myofiber.

Fiber Damage

Damage to the fibers can be seen in multiple ways within the muscle fibers. As muscle is injured, the integrity of the sarcomeres can be compromised, this break of the regular structure can be easily seen in the SHG images as the repeating banding pattern across the fibers will not be present. Besides this obvious visualization, the membrane surrounding the fibers themselves can be compromised, giving the fibers an almost ruffled appearance instead of their normal smooth boundary. Within some of these images hypercontracted muscle fibers can also be seen, these are the fibers that have bunched up as they pull together; however, this is most likely due to the removal of the muscle than from the acute injury that was sustained by the mice.

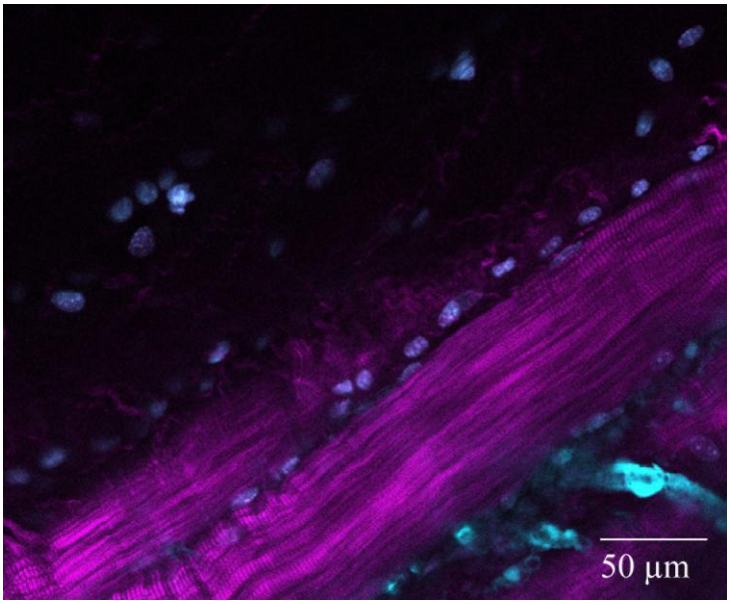


Figure 10- Damaged Region

In this figure a damaged region within the central region of the fiber. This region of damage breaks from the well-ordered sarcomere patterning seen at the ends of this fiber where there is no damage evident.

Discussion

Through the use of SHG imaging the different key stages of muscle regeneration can be clearly visualized with minimal preparation or interference, providing an ideal method for the assessment of key physiological properties. In this experimental set we were clearly able to visualize the activation of the satellite cells and proliferation of nuclei in response to the acute damage caused by CTX-1. We also saw denovo fiber formation and repair through fusion and then the gradual return of the repaired muscle fibers towards their original resting state. This technique has potential to act as a pivotal tool in the future, and with some more development will be able to act as a convenient method to capture a quantitative glimpse of muscle tissue, and hopefully provide insight into muscular damage and regeneration. To start working towards this development, a more detailed time course can be used, providing samples from closer periods within the regenerative procedure which would provide a greater view of the timings involved in the regenerative process. Nuclei migration within the fibers is a key focus of regeneration within skeletal muscle, and creating a method for precise capture of this migration would provide an invaluable tool for muscular studies. Since SHG imaging can be done within live tissues, instead of sacrificing animals at different time points to observe the general mechanisms, a single mouse could be used to provide imaging from within a live repairing tissue, something which will in the future hopefully be applied to human samples. By imaging live tissue, the actual migration of nuclei from within a muscle fiber could be observed, and the kinetics of the individual nuclei within the fiber could be monitored as they migrate from the periphery towards their central location and back out towards their resting locations.

Shnitzer and colleagues have worked on developing tools for the convenient imaging of human samples using micro-optics and two photon fluorescence; so far, they have been able to image a diverse group of tissues ranging such as individual neurons within a brain and muscle tissue(18). By using two-photon fluorescence and fiber based devices they have been able to create truly amazing tools that are able to take advantage of both two photon fluorescence and the advancing technology used to create small-scale imaging devices. Together they have created a small (2.9g) portable two-photon fluorescence microendscope that allows the visualization of tissue to a micrometer scale resolution; this tool can provide amazing benefits to clinical research and applications along with use on small animal studies (18).

In just the field of aging alone this imaging technique provides a much needed tool. With the ability to section larger areas of muscle without the need for fixation or stains, a clinical tool for the evaluation of muscle strength or integrity may be possible. Sarcopenia is very hard to identify as it is a natural process, and has limited cues that could be easily observed. SHG provides an easy method to take a look at the tissue of human patients as it does not require the extensive preparation that most current techniques requires and can be safely used *in vivo*. As in Schnitzer's work, SHG offers possibility of assessing muscle quality without invasive muscle biopsy procedures, which are never performed, unless perhaps in very serious terminal pathologies such as muscular dystrophy or amyotrophic lateral sclerosis. SHG has the potential to identify physiological markers of frail muscle since it is able to provide a much more detailed and reliable quantitative view on the muscle. Factors such as the size or shape of the nuclei, sarcomere integrity or the amount of interstitial expansion accumulated within the muscle over regular regenerative cycles are all properties that can be accurately seen and evaluated with SHG. These factors and more like them may prove to be valuable markers for the identification of sarcopenia in humans. It has been found that the mean number of myonuclei present within the fibers of aging tissue samples remains consistent; however the satellite cell population does not; in aged samples the satellite population drops to 0.6-3.4% of the nuclei population from the normal 4% in adults (2). Another study was conducted that focused more on the shape of the nuclei between young and old mature muscle fibers. From that study it was seen that nuclei of young muscle fibers are both rounded and ellipsoidal and aligned generally with the longitudinal axis of the muscle fibers. In aged adults, the nuclei showed more deviations from the rounded or ellipsoidal shapes with indentations in the nuclear envelope, additionally they were less ordered than the young muscle aggregating together along the fiber (19), If any identifying factors can be found for muscle degeneration it has a great potential to provide early detection for sarcopenia in older adults instead of identifying the degradation of muscle only after it has already affected the mobility of the patient. Besides easier and earlier identification if the physiological aspects of sarcopenia can be identified, it may reveal new methods of treating the muscle degradation. Instead of simply prescribing exercise, it would allow a targeted response towards the cause or effect of the identified physiologic response, something which at the moment doctors are unable to do since only the general trend and not the mechanism of how or why are known.

Muscle related diseases are also a field where this imaging technique may play a vital role in the future. With diseases such as muscular dystrophies may benefit from this technique as a diagnostic tool as well as a research tool for future developments with the treatment of the diseases. Muscular diseases usually depend at least in part on a biopsy, with the ability of two photon fluorescence to image within live tissues it may provide a much less harmful and invasive option for the identification of muscular diseases and skeletal muscle weakness.

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